Kinetics of Basic Fibroblast Growth Factor Binding to Its Receptor and Heparan Sulfate Proteoglycan: A Mechanism for Cooperativity†

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ABSTRACT: Basic fibroblast growth factor (bFGF) binds to cell surface receptor (CSR) proteins and to heparan sulfate proteoglycans (HSPG). On the basis of equilibrium dissociation constants (K_d), the CSR has been considered a "high-affinity" binding site and HSPG a "low-affinity" site. We measured the apparent individual *on* and *off* rate constants (k_{on} and k_{off}) for bFGF binding to these two sites on intact cells and to each class of binding site in the absence of the other. While the k_{on}'s for CSR and HSPG on intact cells were not statistically different ($k_{on}^C = 2.27 \times 10^8$ M^{-1} min^{-1}; $k_{on}^H = 0.90 \times 10^8$ M^{-1} min^{-1}), the k_{off} for the HSPG was 22.7-fold greater than that for the CSR ($k_{off}^C = 0.003$ min^{-1}; $k_{off}^H = 0.68$ min^{-}). Thus, the difference in K_d's appears to result from the faster rate at which bFGF is released from the HSPG sites compared to the CSR. The k_{on}'s for isolated CSR and HSPG, and the k_{off} for isolated HSPG, did not differ significantly from those for intact cells $k_{on}^C = 2.50 \times 10^8$ M^{-1} min^{-1}; $k_{on}^H = 0.92 \times 10^8$ M^{-1} min^{-1}; $k_{off}^H = 0.095$ min^{-1}). However, the *off* rate for isolated CSR ($k_{off}^C = 0.048$ min^{-1}) was statistically indistinguishable from the off rate for HSPG and 16-fold greater than the *off* rate for CSR on intact cells. The "high-affinity" binding of bFGF to intact cells probably refers only to a complex of bFGF with both CSR and HSPG, and not to the CSR alone.

Basic fibroblast growth factor (bFGF)[1] is a 16–25-kDa protein that stimulates the proliferation of a number of cell types in vitro, including cells of epithelial and mesenchymal origin, and bFGF is proposed to be of general importance in the development and maintenance of complex biological structures [see Klagsbrun (1989), Rifkin and Moscatelli (1989), Burgess and Maciag (1989), and Baird and Bohlen (1990) for reviews].

The cell surface receptors (CSR) for bFGF have been identified and are considered "high-affinity" binding sites on the basis of the low K_d values ($K_d = 10^{-9}$–10^{-12} M) (Neufeld & Gospodarowicz, 1985; Moenner et al., 1986; Olwin & Hauschka, 1986). The biological activity of bFGF is believed to be mediated by the interaction with these receptors, and binding of bFGF to the CSR stimulates tyrosine kinase activity and phosphorylation of the CSR (Huang & Huang, 1986; Coughlin et al., 1988). A lower affinity ($K_d = 10^{-8}$–10^{-9} M) interaction of bFGF with cells has also been observed (Moscatelli, 1987). These binding sites have been determined to be heparan sulfate proteoglycans (HSPG) on the cell surface and in the extracellular matrix (Vlodavsky et al., 1987; Moscatelli, 1988; Bashkin et al., 1989). The number of these lower affinity bFGF binding sites on cells in culture is generally 1–3 orders of magnitude greater than the number of CSR (Moscatelli, 1987; Burgess & Maciag, 1989 for review).

The bFGF–HSPG interaction is believed to be representative of the well-characterized heparin affinity of bFGF which facilitated the purification of bFGF (Shing et al., 1984; Klagsbrun & Shing, 1985). The binding of bFGF with heparin has been found to protect bFGF from degradation and denaturation (Gospodarowicz & Cheng, 1986; Sommer & Rifkin, 1989). In addition, the interaction of bFGF with endothelial cell-derived heparan sulfate proteoglycans (HSPG) results in resistance of bFGF to proteolytic degradation (Saksela et al., 1988), and may increase the radius of bFGF diffusion (Flaumenshaft et al., 1990). Endogenous bFGF has been found associated with heparin-like molecules in the subendothelial ECM in cell culture and with basement membranes in vivo (Vlodavsky et al., 1987; Folkman et al., 1988; Gonzalez et al., 1990). In addition, the ECM has been implicated as a source of bFGF for long-term stimulation of DNA synthesis in bovine capillary endothelial cells, for growth of vascular endothelial cells, and for the differentiation of PC12 cells (Rogelj et al., 1989). These findings suggest that the HSPG in the ECM may serve as a reservoir of bFGF, releasing it for interaction with cell surface receptors.

The role of HSPG binding of bFGF has recently been investigated (Yayon et al., 1991; Rapraeger et al., 1991; Heath et al., 1991). Yayon et al. (1991) demonstrated that binding of bFGF to the CSR requires the presence of heparan sulfate or heparin by examining bFGF binding to a series of Chinese hamster ovary cell lines with varying abilities to produce proteoglycan and engineered to express bFGF receptors. Rapraeger et al. (1991) demonstrated that binding and biological activity of bFGF on fibroblasts and myoblasts required heparan sulfate. Heath et al. (1991) observed that mutations in the heparin binding domains of bFGF altered the mitogenic activity of bFGF on human fibroblasts. These

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[1] Abbreviations: bFGF, basic fibroblast growth factor; CSR, cell surface receptor(s); DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; HSPG, heparan sulfate proteoglycan(s); K_d, equilibrium dissociation constant; k_{on}, association rate constant; k_{off}, dissociation rate constant; PBS, phosphate-buffered saline; se, standard error of the mean.

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results suggested to the authors that heparan sulfate binding may act as a bFGF shuttle to facilitate bFGF interaction with and activation of bFGF–receptors. A mechanism requiring bFGF to bind first to HSPG and then to the CSR seems counterintuitive since the equilibrium dissociation constant for the HSPG is much larger than that for the CSR, suggesting that bFGF binding to the HSPG would occur only after binding to the CSR was saturated.

The K_d's alone might not provide a complete understanding of the nature and the potential interaction between these two classes of binding. By definition, the equilibrium dissociation constant is equal to the quotient of the dissociation rate constant, k_{off}, and the association rate constant, k_{on}. We now define the individual apparent k_{on}'s and k_{off}'s for bFGF binding to CSR and HSPG by following the kinetics of bFGF association and dissociation on intact Balb/c3T3 cells, on cells treated with heparinase to selectively remove the bFGF binding HSPG, and to detergent-extracted HSPG in the absence of cells. The observed k_{on}'s for these two classes of binding sites on intact cells were not significantly different, but the apparent k_{off}'s for the two sites differed by more than 20-fold. While the binding of bFGF to isolated HSPG was not different than to HSPG on intact cells, bFGF binding to isolated CSR was decreased as a result of an increase only in the *off* rate. Therefore, heparan sulfate mediated regulation of bFGF binding to its CSR probably does not involve alteration in the association of bFGF to the CSR but rather stabilization of the CSR–bFGF complex. Our results suggest that "high-affinity" bFGF binding to intact cells may reflect a ternary complex consisting of bFGF bound to both CSR and HSPG, and not to CSR alone. These results may help further the definition and understanding of what have been termed "high-" and "low-affinity" receptors for bFGF.

MATERIALS AND METHODS

Cells. Mouse Balb/c3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in 100-mm culture dishes (Corning, Corning, NY) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL) (Gibco), and 10% calf serum (Colorado Serum Co., Denver, CO). Subculturing was carried out with 0.05% trypsin with 0.53 mM EDTA in Hank's balanced salt solution (Gibco) when the cells were subconfluent. Fresh vials of cells were thawed every 2 months. Cell numbers were determined by counting trypsinized cells with a Coulter counter.

Confluent Balb/c3T3 cells were used for all experiments. Confluent monolayers were prepared by plating 5×10^4 cells per well in 24-well plates, 2 cm^2 wells (Costar), in DMEM–10% calf serum (1 mL/well) and incubating (at 37 °C) for 3–5 days. For ^{125}I-bFGF cross-linking experiments, confluent monolayers in 100-mm dishes were used in which 5×10^5 cells per dish were plated in 10 mL of DMEM–10% calf serum. For extracellular matrix isolation, and bFGF binding to ECM-coated dishes, confluent monolayers were prepared in 6-well plates by plating 2.5×10^5 cells per well in DMEM–10% calf serum.

Materials. Pure human recombinant bFGF (18 kDa) was from Chiron Inc. (Emeryville, CA). bFGF was stored in buffer containing 50 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1 mM dithiothreitol, and 0.05% gelatin, to prevent dimerization and inactivation. Gelatin and Hepes were from Sigma (St. Louis, MO). ^{125}I-bFGF binding kinetic analysis was done with KINETIC from Biosoft (Milltown, NJ) using a Macintosh SE computer. Heparinase (Hep I), EC 4.2.2.7 (1.67 units/μg), was purified from *Flavobacterium heparinum*; it eluted as a single peak in reverse-phase HPLC, produced a single band on silver-stained SDS–polyacrylamide gels, and was found to be free of other lyase activity (Yang et al., 1985). Heparitinase was a generous gift from Dr. Arthur Lander (Massachusetts Institute of Technology).

Radiolabeling of bFGF. ^{125}I-bFGF was prepared by a modification of the Bolton–Hunter procedure (Bashkin et al., 1989; Kurokawa et al., 1989). This technique has been demonstrated to produce active ^{125}I-bFGF as determined by its ability to bind heparin–Sepharose and to stimulate DNA synthesis in Balb/c3T3 and bovine capillary endothelial cells (Bashkin et al., 1989; Kurokawa et al., 1989). Lyophilized bFGF was dissolved in 100 mM sodium phosphate buffer, pH 8.5 (final concentration of 250–350 μg/mL), and an aliquot (30 μL) was immediately added to dry Bolton–Hunter reagent (1 mCi, 0.6 μmol; New England Nuclear, Boston, MA) and incubated on ice for 2.5 h. The reaction was quenched by adding 200 μL of 0.2 M glycine and incubating on ice for 45 min. Twenty microliters of 0.5% gelatin was added followed by the addition of 250 μL of gel filtration buffer (50 mM Tris-HCl, 0.05% gelatin, 1 mM dithiothreitol, and 0.3 M NaCl, pH 7.5). The combined sample (0.5 mL) was then applied to a PD-10 column (bed volume 10 mL; Pharmacia LKB Biotechnology, Piscataway, NJ) equilibrated with gel filtration buffer; 25–40% of the radioactivity that eluted from the column was in the initial peak (1–2.5 mL). When this peak was run on SDS–PAGE gels (4–14% gradient), 94.0 ± 0.5% of the cpm was localized to a single band with an apparent molecular weight of 18K, equivalent to that of unlabeled bFGF. The specific activity of the ^{125}I-bFGF was assessed by stimulation of quiescent Balb/c3T3 cells and was 25–100 μCi/μg. In this assay, half-maximal DNA synthesis is observed in the presence of 0.05–0.2 ng of untreated bFGF or 0.01–0.05 μL of the ^{125}I-bFGF column fraction.

^{125}I-bFGF Binding. ^{125}I-bFGF binding was conducted with confluent Balb/c3T3 cells to determine the number and kinetic characteristics of the HSPG and CSR bFGF binding sites (Moscatelli, 1988; Bashkin et al., 1989). Prior to the initiation of ^{125}I-bFGF binding, the monolayers were washed once with 1 mL of 4 °C binding buffer (DMEM–25 mM Hepes, pH 7.4, containing 0.05% gelatin) and then incubated for 10 min at 4 °C in 0.5 mL of binding buffer to precool the monolayers. ^{125}I-bFGF [5 ng, 0.55 nM, $(1.25–5) \times 10^5$ cpm] was then added directly to each well.

^{125}I-bFGF Association Kinetics. Association was determined by incubating the cells with ^{125}I-bFGF for the indicated time, at which point unbound ^{125}I-bFGF was removed by immediate removal of the binding buffer and three rapid washes with cold binding buffer (1 mL $well^{-1}$ $wash^{-1}$). The amount of ^{125}I-bFGF bound to HSPG and to CSR was determined sequentially in each culture using a modification of the salt/acid washing technique described by Moscatelli (1987). The ^{125}I-bFGF bound to the HSPG was released by exposure to high-salt buffer (2 M NaCl–20 mM Hepes, pH 7.4; 0.5 mL/well for 5 s), and then the CSR-bound ^{125}I-bFGF was extracted with 5-min incubation of the monolayers in low-pH buffer (2 M NaCl–20 mM sodium acetate, pH 4.0; 0.5 mL/well) followed by a wash with the same buffer (0.5 mL/well). Labeled bFGF was determined in all samples by counting in a 1272 CliniGamma γ counter (LKB Nuclear, Gaithersburg, MD). The ^{125}I-bFGF binding that was not competed by an excess (5 μg; 555 nM) of unlabeled bFGF was defined as nonspecific and was subtracted from the experimental points. Nonspecific binding was measured at each time point and was found to increase without saturation

as a first-order process over the course of the experiment for both the high-salt and low-pH washes. Nonspecific binding was between 5 and 15% of the total binding when binding was maximal. The number of cells attached to the culture plates before and after the salt and acid washes was not different.

Salt and Acid Wash. The ability of the salt and acid washes to distinguish between CSR and HSPG was determined by conducting bFGF binding studies at several different bFGF concentrations. The amount of bFGF released by the salt and acid washes agreed closely to the amount predicted to bind to these sites based on independent Scatchard analysis (Nugent & Edelman, 1992). For instance, when the binding was conducted with low concentrations of bFGF, most of the bound bFGF was released during the acid wash and not the salt wash, consistent with predominently CSR binding. However, when cells, bound with ^{125}I-bFGF, were incubated for longer periods (1–10 min) with the high-salt solution, a treatment expected to remove exclusively HSPG binding (Moscatelli, 1987, 1988; Yayon et al., 1991), a significant portion of the CSR-bound growth factor was released (data not shown). This suggested that HSPG and CSR binding on intact cells may not be completely independent and that a considerable amount of physical dissociation of bFGF–CSR may occur within the 1–10-min incubation when bFGF–HSPG complexes are destabilized. As a practical matter, to ensure that salt washing removed only HSPG-bound bFGF, the minimum incubation period with the high-salt solution required to completely release all the HSPG-bound bFGF was determined using detergent-extracted HSPG which contained no CSR. The high-salt wash may theoretically contain a small fraction of CSR-bound bFGF, but the growth factor released by the acid wash is unlikely to contain much HSPG-bound bFGF.

^{125}I-bFGF Dissociation Kinetics. To determine dissociation kinetics, the cells were incubated with ^{125}I-bFGF (0.55 nM) at 4 °C until binding reached an equilibrium level (3 h). At this point, the binding buffer containing ^{125}I-bFGF was removed, and the monolayers were washed 3 times with ice-cold binding buffer. Then 1.0 mL of 4 °C binding buffer containing unlabeled bFGF (1.0 μg; 55 nM) was added to each well, and the cells were incubated at 4 °C for the indicated time. Unlabeled bFGF was included in the dissociation medium to ensure that released ^{125}I-bFGF would not rebind to unoccupied receptors. However, the possibility that microrelease and rebinding occur under these conditions cannot be completely ruled out. After the dissociation period, the binding buffer was removed, the cells were washed once with cold binding buffer, and the amount of ^{125}I-bFGF that remained bound to the HSPG and CSR was determined with salt and acid as described above. The amount of ^{125}I-bFGF bound to nonspecific sites was determined by including bFGF (5 μg; 555 nM) in the buffer during the 3-h binding incubation, and represented 5–15% of the total ^{125}I-bFGF bound at this point. The amount of ^{125}I-bFGF in the salt and acid washes from these wells was determined at each time point and subtracted from the experimental points.

^{125}I-bFGF Equilibrium Binding and Cross-Linking. For equilibrium binding studies, the cells were incubated with ^{125}I-bFGF at 4 °C for 3 h and then washed, and the bound ^{125}I-bFGF was determined immediately with the salt/acid procedure. For receptor cross-linking studies, the unbound ^{125}I-bFGF was removed after a 3-h binding incubation, the monolayers were washed an additional 3 times with PBS, and bound ^{125}I-bFGF was cross-linked to its binding sites by incubating the cells with 0.15 mM disuccinimidyl suberate (Pierce, Rockford, IL) in PBS for 30 min at 4 °C (Kurokawa et al., 1989). The ^{125}I-bFGF-cross-linked cells were then extracted by scraping with a Teflon cell lifter (Costar, Cambridge, MA) into boiling SDS–PAGE sample buffer and analyzed on 5% polyacrylamide gels. The ^{125}I-bFGF-cross-linked proteins were visualized by autoradiography, and the relative intensity of each band was determined using an LKB UltroScan XL laser densitometer.

Heparinase Treatment. In some experiments, the Balb/c3T3 cells were treated with heparinase prior to ^{125}I-bFGF binding. Confluent Balb/c3T3 cells were washed once with 37 °C DMEM and then treated with heparinase (1 μg/mL) in DMEM for 15 min at 37 °C. The heparinase and soluble degradation products were removed by washing the cells 3 times with 4 °C binding buffer, and ^{125}I-bFGF binding was carried out as described above. The treatment was determined to be sufficient for heparinase to go to completion, as longer incubation with higher heparinase concentrations did not result in increased digestion of ^{35}S-proteoglycans produced by these cells (data not shown). In addition, the heparan sulfate specific enzyme heparitinase gave similar results. Treatment of the cells with heparinase was not cytotoxic. Cell viability and total cell number were not different in heparinase-treated compared to untreated cells. Cell viability was determined by trypan blue exclusion and found to be 96.2 ± 0.5% and 96.9 ± 1.7% in untreated and heparinase-treated cells, respectively.

Extracellular Matrix Preparation. ECM-coated plates were prepared by solubilizing confluent Balb/c3T3 cells with PBS containing 0.5% Triton X-100 and 20 mM NH_4OH for 3 min at room temperature, followed by 4 washes with PBS (Vlodavsky et al., 1987; Bashkin et al., 1989). While ECM prepared in this way has been demonstrated to contain ECM elements, it has also been shown to retain some cellular elements such as intracellular histones (Gajdusek & Carbon, 1989). This material is referred to as ECM operationally since the presence of HSPG and not CSR is observed (Bashkin et al., 1989). However, it is not known to what extent the HSPG in the ECM preparation represents HSPG originally from the ECM, the plasma membrane, or both. ^{125}I-bFGF binding to the isolated ECM was carried out as described above for intact cells.

BINDING KINETIC CONCEPTS

The kinetics of bFGF binding to CSR and HSPG in Balb/c3T3 cell cultures were measured on living cells assuming two independent processes:

$$\text{bFGF} + \text{CSR} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{bFGF–CSR} \quad (1)$$

$$\text{bFGF} + \text{HSPG} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{bFGF–HSPG} \quad (2)$$

where the dissociation constant (K_d) for each reaction is equal to the ratio of the *off* rate constant (k_{off}) and the *on* rate constant (k_{on}) and is related to the reactions in eq 1 and 2 as

$$K_d = \frac{k_{off}}{k_{on}} = \frac{[\text{bFGF}][\text{R}]}{[\text{bFGF–R}]} = \text{molar unit} \quad (3)$$

where [R] represents [HSPG] or [CSR].

On the basis of the K_d values, the CSR has been considered to be a "high-affinity" binding site and the HSPG a "low-affinity" site. We investigated this difference in K_d's dy-

namically at the kinetic level. The binding kinetics to each class of site are approximated by the expressions:

$$\frac{d[bFGF\text{–}R]}{dt} = k_{on}[bFGF][R] - k_{off}[bFGF\text{–}R] \quad (4)$$

$$\text{association rate} = k_{on}[bFGF][R] \quad (5)$$

$$\text{dissociation rate} = k_{off}[bFGF\text{–}R] \quad (6)$$

where $[R] = [R]_{tot} - [bFGF\text{–}R]$.

From the association data, the quantity of bound bFGF obtained versus time can be transformed to meet the equation of a straight line:

$$\ln \frac{[bFGF\text{–}R]_{eq}}{([bFGF\text{–}R]_{eq} - [bFGF\text{–}R])} = (k_{off} + k_{on}[bFGF])t \quad (7)$$

$$k_{obs} = k_{off} + k_{on}[bFGF] \quad (8)$$

Using this manipulation, k_{obs} was determined graphically, or, conversely, the k_{obs} was generated using a computer curve-fitting program. Then the association rate constant, k_{on} (min^{-1} M^{-1}), was determined from the k_{obs} and the k_{off}. The k_{off} was determined independently by measuring the dissociation under conditions in which association was prevented. This was done by removing the unbound (free) ^{125}I-bFGF and including an excess of unlabeled bFGF in the dissociation buffer. Thus, the concentrations of ^{125}I-bFGF ([bFGF]) and free receptors ([R]) were effectively zero, such that the amount of bound ^{125}I-bFGF decreased with time according to the equation:

$$\frac{d[bFGF\text{–}R]}{dt} = -k_{off}[bFGF\text{–}R] \quad (9)$$

The amount of ^{125}I-bFGF bound at any time t will relate to the amount initially bound ($t = 0$) and to the k_{off} as shown:

$$\ln \frac{[bFGF\text{–}R]_t}{[bFGF\text{–}R]_{t_0}} = -k_{off}(t) \quad (10)$$

bFGF binding kinetics were measured on both sites together (intact cells) or on each site individually, in the absence of the other (heparinase-treated cells = CSR alone; ECM-coated dishes = HSPG alone).

Statistics and Error Estimation. All binding data are expressed as the average ± standard error about the mean. Densitometric analyses of radiolabeled protein bands are presented as average relative units ± standard deviation. Statistical comparisons were performed with the nonpaired t test for groups of unequal sample sizes or two-way analysis of variance when appropriate. Data were rejected as not statistically significantly different if p values of greater than 0.05 were observed. The apparent binding constants were determined by nonlinear least-squares curve-fitting to theoretical binding behavior using the computer curve-fitting program KINETIC. While the precision is good in data from individual triplicates throughout a single experiment, all kinetic values presented are the average values from three experiments, so the uncertainty in these values is cumulative. The exact confidence limits to the data were not determined; rather, we have estimated the extremes of the possible values. This method of error estimation results in relatively large errors, especially for the k_{on} values since these include the combined experimental error in the k_{obs} and k_{off}. This approach was used since we do not understand all potential sources for variation and because none of the conclusions derived from these data is dependent on a more precise determination of the error limits. The calculated K_d values are a ratio of k_{off} and k_{on}; hence, the range is presented as a multiple factor, based on the extremes for the individual constants, rather than an absolute value. The data presented in the figures are from single experiments.

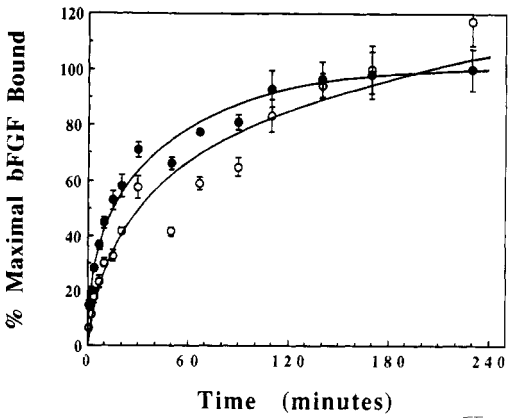

FIGURE 1: bFGF association to CSR and HSPG on intact Balb/c3T3 cells. Confluent cultures of Balb/c3T3 cells were incubated with ^{125}I-bFGF for the indicated time, and then the amount bound to the CSR (●) and HSPG (○) was determined. Each point represents the average ± se of triplicate wells. Maximal binding (100%) was 6491 ± 314 cpm for CSR and 8598 ± 465 cpm for HSPG. The average *on* rate constants for the CSR and HSPG were 2.27 (±1.11) × 10^8 and 0.90 (±0.51) × 10^8 M^{-1} min^{-1} respectively (p = NS).

RESULTS

Rate Constants for bFGF Binding to Cell Surface Receptors and Heparan Sulfate Proteoglycans on Balb/c3T3 Cells. To investigate the nature of the interaction of bFGF with CSR and HSPG, we measured the association and dissociation kinetics for bFGF binding to Balb/c3T3 cells. The bFGF association kinetic profiles produced for CSR and HSPG were almost identical (Figure 1). The k_{on}'s for bFGF binding to CSR (2.27 × 10^8 M^{-1} min^{-1}) and HSPG (0.90 × 10^8 M^{-1} min^{-1}) were not statistically significantly different. However, it has been observed that there are 10–1000 times as many HSPG binding sites than CSR on a variety of cells from several species [see Moscatelli (1987) and Burgess and Maciag (1989) for a review]. We have confirmed this with our Balb/c3T3 cells by Scatchard analysis and found a 100-fold greater amount of HSPG than CSR (Nugent and Edelman, unpublished results). Therefore, if [HSPG] = 100[CSR] and $k_{on}^{C} \cong k_{on}^{H}$, then the *on* rate of bFGF should be 100 times greater for the "low-affinity" HSPG than for the "high-affinity" CSR (see eq 5).

The bFGF dissociation kinetics from these two types of binding sites differed significantly; the k_{off} for the HSPG (0.068 min^{-1}) was 22.7 times greater than that for the CSR (0.003 min^{-1}) ($p < 0.0005$) (Figure 2). Thus, the time required for half of the bound bFGF to be released ($t_{1/2}$) from the CSR was 150–300 min compared to 5–20 min for the HSPG. The majority of the salt-extractable bFGF dissociated within 60 min, and the remainder was released at a slower rate (k_{off} = 0.005 min^{-1}), consistent with the demonstration that the salt wash contains a fraction of CSR-bound bFGF. Therefore, the calculated value for the HSPG k_{off} is likely to be an underestimate, the k_{off} for the CSR is probably a slight overestimate, and the difference between the k_{off}'s for these two sites may actually be greater than reported. The dissociation constants calculated from these rate constants were within the range of those previously reported on the basis of equilibrium binding and Scatchard analysis [see Klagbrun (1989), Rifkin and Moscatelli (1989), Burgess and Maciag (1989), and Baird and Bohlen (1990) for reviews]. The K_d for HSPG (0.76 nM) was higher than that for CSR (0.01 nM), and, on the basis of the experimental errors for

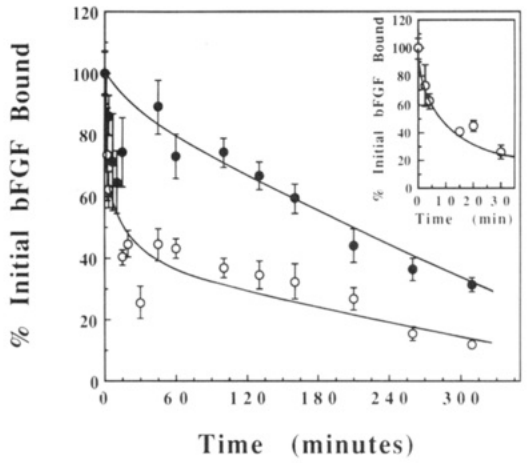

FIGURE 2: bFGF dissociation from CSR and HSPG on intact Balb/c3T3 cells. ^{125}I-bFGF was bound to confluent cultures of Balb/c3T3 cells, and then the unbound ^{125}I-bFGF was removed. The release of ^{125}I-bFGF from CSR (●) and HSPG (○) was followed by measuring the amount remaining bound at each time point. The inset shows the early time points for bFGF dissociation from HSPG. The majority of bFGF dissociated from these sites in the first 30 min with the remainder dissociating at a slower rate. Dissociation was performed in the presence of an excess of unlabeled (1 μg; 55 nM) bFGF to minimize rebinding of released ^{125}I-bFGF. Each data point is the average ± se of triplicate wells. Initial binding (100%) was 2337 ± 106 cpm for CSR and 1626 ± 8 cpm for HSPG. The average *off* rate constant for the CSR was 0.003 ± 0.001 min^{-1}, and the majority of the bFGF defined as HSPG-bound released with an *off* rate constant of 0.068 ± 0.028 min^{-1}, with the remainder being released at a slower rate (0.005 ± 0.002 min^{-1}). The kinetics of bFGF dissociation from CSR and HSPG were statistically significantly different ($p < 0.0005$).

Table I: Equilibrium Binding of bFGF to CSR and HSPG on Heparinase-Treated Balb/c3T3 Cells[a]

	^{125}I-bFGF bound		
	control	heparinase treated	
	cpm	cpm	% of control
CSR	4474 ± 70	1198 ± 38	26.8 ± 0.9
HSPG	6460 ± 88	374 ± 64	5.8 ± 1.0

[a] ^{125}I-bFGF binding to CSR and HSPG on untreated and heparinase-treated Balb/c3T3 cells was carried out at 4 °C for 3 h as described under Materials and Methods. The data are the average ± se of two separate experiments which were each carried out in triplicate. Treatment of the cells with heparitinase resulted in a similar reduction in bFGF binding to HSPG (3.7 ± 2.6% of control) and CSR (28.2 ± 5.5% of control).

the individual kinetic contants, the error in the calculated K_d values ranged by a factor of approximately 2.5.

Removal of HSPG with Heparinase Alters bFGF Binding to CSR. It has been postulated that HSPG might act as accessory molecules to deliver bFGF to the CSR [see Yayon et al. (1991), Rapraeger et al. (1991), and Klagsbrun and Baird (1991) for a review]. To determine if the presence of HSPG affects the binding of bFGF to the CSR, we treated cells with heparinase to degrade the HSPG prior to bFGF binding. Equilibrium binding of bFGF to heparinase-treated and control (not treated) cells was conducted, and the amount of bFGF bound to CSR and HSPG was determined (Table I). Heparinase treatment resulted in almost complete loss of HSPG binding (5.8% of that on non-enzyme-treated cells). When longer treatment periods with higher concentrations of heparinase were conducted, similar results were observed (data not shown). Thus, the residual salt-sensitive binding most likely represents CSR-bound bFGF which is sensitive to the salt treatment. However, the possibility that this represents a heparinase-resistant subclass of HSPG cannot be formally ruled out at this time. In addition to decreasing HSPG binding,

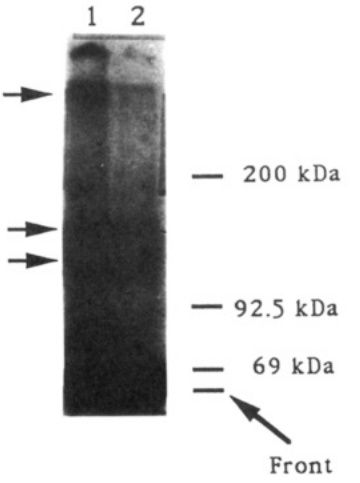

FIGURE 3: bFGF affinity cross-linking to untreated and heparinase-treated Balb/c3T3 cells. Equilibrium binding of ^{125}I-bFGF was carried out with untreated (lane 1) and heparinase-treated (lane 2) cells. After the binding period, bound ^{125}I-bFGF was cross-linked to CSR and HSPG sites with disuccinimidyl suberate and analyzed by gel electrophoresis and autoradiography. The relative intensities of the three major cross-linked bands were quantitated by averaging the integrated areas of each peak from three separate laser traces: the center and just to the right and left of center in each lane. The high molecular weight band had a relative intensity of 1.29 ± 0.04 (lane 1) and 0.017 ± 0.004 (lane 2); the 150-kDa band, 0.81 ± 0.02 (lane 1) and 0.43 ± 0.06 (lane 2); the 125-kDa band, 1.57 ± 0.04 (lane 1) and 0.72 ± 0.01 (lane 2). The three bands marked with arrows were not apparent when the binding was done in the presence of an excess of unlabeled bFGF (5 μg; 110 nM).

heparinase treatment caused a significant decrease in bFGF binding to CSR (26.8% of control). This result is consistent with those of Rapraeger et al. (1991) in which treatment of Swiss 3T3 cells with heparitinase or chlorate reduced binding to the CSR by 66% or 88%, respectively.

To visualize the effect of heparinase on the bFGF binding sites, bFGF was cross-linked to the surface of heparinase-treated and nontreated Balb/c3T3 cells (Figure 3). Three major labeled bands were observed on SDS–polyacrylamide gels. A high molecular weight band, similar to that which has previously been identified as bFGF binding HSPG on several cells including Balb/c3T3 cells (Saksela et al., 1988; Vigny et al., 1988; Nugent & Edelman, 1992), was found in control but was almost completely absent on heparinase-treated cells (relative intensity 1.3 ± 0.3% of control). Two additional bands which migrated with apparent molecular weights of 125K and 150K were found in both control and heparinase-treated cells. These labeled bands are similar to the bFGF cell surface receptors previously identified on several cells [see Neufeld and Gospodarowicz (1985), Moenner et al. (1986), Olwin and Hauschka (1986), and Burgess and Maciag (1989) for a review]. Labeling of these two bands was less intense in the heparinase-treated cells; the relative intensity of the 125-kDa band was 46 ± 0.5% of the control and of the 150-kDa band was 53 ± 7% of the control. These results are consistent with the decrease in bFGF binding to the CSR, and no evidence of receptor degradation was observed. Therefore, the binding of bFGF to CSR seems dependent on the presence of bFGF binding HSPG.

Rate Constants for bFGF Binding to Isolated CSR and HSPG. To determine the manner in which HSPG regulates bFGF binding to the CSR, bFGF binding kinetics were measured on heparinase-treated cells (CSR alone) and with extracellular matrix-coated dishes (HSPG alone). When bFGF association kinetics were measured, the *on* rate constants for each site were similar to those observed with intact cells

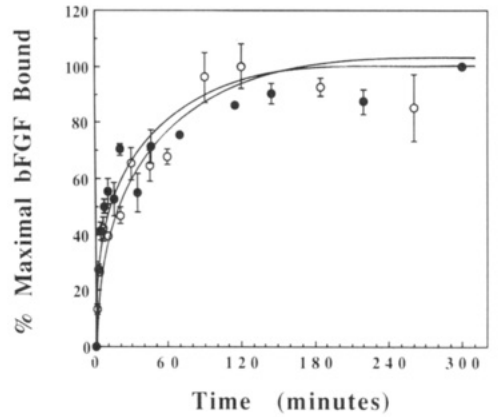

FIGURE 4: bFGF association to isolated CSR and isolated HSPG. Association kinetics for ^{125}I-bFGF to isolated CSR (●) were conducted with heparinase-treated Balb/c3T3 cells. ^{125}I-bFGF binding to HSPG (○) was performed with Balb/c3T3-produced extracellular matrix coated tissue culture plates. The resulted presented are from two separate experiments. Each data point is the average ± se of triplicate wells. Maximal binding (100%) was 2862 ± 20 cpm for isolated CSR and 11 630 ± 1027 cpm for isolated HSPG. The average *on* rate constants for the CSR and HSPG were 2.50 (±0.84) × 10^8 and 0.92 (±0.46) × 10^8 M^{-1} min^{-1}, respectively (p = NS).

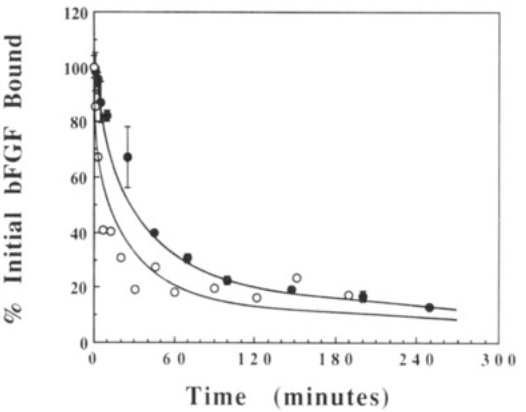

FIGURE 5: bFGF dissociation to isolated CSR and extracellular matrix HSPG. ^{125}I-bFGF was bound to confluent cultures of heparinase-treated Balb/c3T3 cells (isolated CSR) and to extracellular matrix coated tissue culture plates (isolated HSPG). The unbound ^{125}I-bFGF was removed, and the release of ^{125}I-bFGF from CSR (●) and HSPG (○) was followed by measuring the amount remaining bound at each time point. The results are from two separate experiments. The data are the average ± se of triplicate samples. Initial binding (100%) was 2213 ± 101 cpm for isolated CSR and 6109 ± 219 cpm for isolated HSPG. The average *off* rate constants for the CSR and HSPG were 0.048 ± 0.009 and 0.095 ± 0.025 min^{-1}, respectively (p = NS).

(Figures 1 and 4). Similarly, the *off* rate constant for isolated HSPG was not statistically significantly different than that on intact cells (Figures 2 and 5). Although we cannot determine what proportion of the HSPG in the Triton/NH_4-OH-extracted ECM was derived from the original ECM or the plasma membrane, we can conclude that the ECM contained little or no normally functioning CSR since >95% of the ^{125}I-bFGF bound to the ECM was released by the high-salt wash. While bFGF binding to HSPG was not dependent on the presence of CSR, CSR binding was significantly different in the presence of HSPG. The *off* rate constant for the CSR on heparinase-treated cells was 16-fold greater than that for the CSR on intact cells ($p < 0.001$) (Figures 2 and 5). The *off* rate constant for CSR binding in the absence of HSPG was closer to that for the isolated HSPG than the "high-affinity" binding on heparan sulfate containing cells. The higher K_d for CSR in the absence of HSPG (0.19 vs 0.01 nM) was the result of an increase in the *off* rate. This result

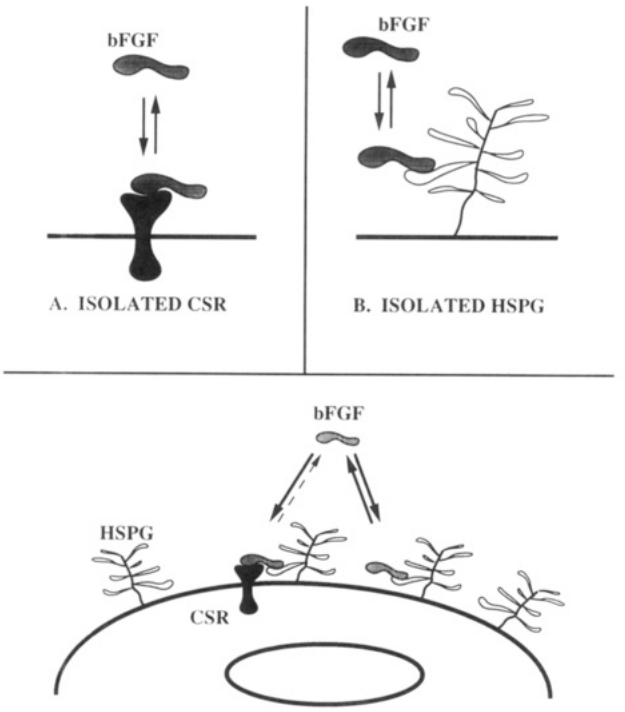

FIGURE 6: Ternary complex model for "high-affinity" bFGF binding. (A) ISOLATED CSR represents heparinase-treated cells containing cell surface receptor proteins without HSPG. (B) ISOLATED HSPG represents Triton/NH_4OH-extracted ECM containing bFGF binding HSPG and no cell surface bFGF receptor proteins. (C) INTACT CELLS – CSR AND HSPG represents cells containing both bFGF receptor proteins and bFGF binding HSPG with an excess of HSPG compared to CSR. "High-affinity" binding is represented as a ternary complex with bFGF bound simultaneously to CSR and HSPG. "Low-affinity" binding is represented as either bFGF binding interaction alone (bFGF–CSR; bFGF–HSPG). The model is not meant to suggest that the bFGF binding HSPG must be plasma membrane bound.

can explain part of the observed decrease in equilibrium bFGF binding to the CSR on heparinase-treated cells (Table I).

DISCUSSION

"High-" and "low-affinity" binding of bFGF has been observed on several cells [see Klagsbrun (1989), Rifkin and Moscatelli (1989), Burgess and Maciag (1989), and Baird and Bohlen (1990) for reviews]. While the "low-affinity" high-capacity binding component has been identified as HSPG (Moscatelli, 1987, 1988; Bashkin et al., 1989), the "high-affinity" component has been identified as CSR (Neufeld & Gospodarowicz, 1985; Moenner et al., 1986; Olwin & Hauschka, 1986). It has recently been determined by several experimental approaches that "high-affinity" CSR binding depends on the presence of HSPG (Yayon et al., 1991; Rapraeger et al., 1991; Table I). The difference between the "high-affinity" binding component and the "low-affinity" component results from a slower *off* rate of bFGF from the "high-affinity" site (Figure 2). A likely possibility is that the slow *off* rate of bFGF with the "high-affinity" sites on intact cells is a result of a complex formed in which bFGF is bound to both CSR and HSPG binding sites simultaneously, producing a more stable complex than either interaction alone (Figure 6). It is interesting to note that the product of the *off* rate constant for isolated CSR and that for isolated HSPG is approximately equal to that for the "high-affinity" CSR on intact cells. This might be interpreted as a stochastic process. The probability that a bFGF molecule will dissociate from a

"high-affinity" CSR, on an intact cell, appears equal to the probability of the simultaneous dissociation of a bFGF molecule from both an isolated CSR and an HSPG binding site. This model is consistent with previous structural analyses of bFGF, which have suggested the existence of separate heparin binding and receptor binding domains on bFGF (Baird et al., 1988; Kurokawa et al., 1989; Eriksson et al., 1991; Zhang et al., 1991; Zhu et al., 1991).

Three distinct classes of bFGF binding may exist on cells, bFGF–CSR, bFGF–HSPG, and CSR–bFGF–HSPG, and only the ternary complex might represent what has been previously termed the "high-affinity" binding sites. The relative amount of the three types of binding complexes would then depend on the ratio of heparan sulfate to CSR. For example, on cells such as those used in this study, which express a 100-fold molar excess of HSPG to CSR, we would expect to see only two classes of binding: bFGF–HSPG and CSR–bFGF–HSPG. The presence of HSPG might act in a cooperative manner to prolong the period in which bFGF is bound to the CSR. Although dependence on the period of bFGF–receptor occupancy for activation is not known, it is tempting to speculate that receptor activation requires a minimum period of occupancy that is not achieved in the absence of HSPG.

The inherent K_d for the CSR, in the absence of HSPG (0.19 nM), is not different than what has been termed a "low-affinity" binding site [see Burgess and Maciag (1989) for a review]. Although the bFGF–HSPG interaction is of higher capacity and of greater statistical probability, it is less stable than the bFGF–CSR interaction on intact cells since the *off* rate from the HSPG is much greater than that for the CSR. A role for the HSPG as accessory molecules that act as primary bFGF binding sites which concentrate and deliver bFGF to the CSR is supported by the binding kinetics (Figures 1 and 2). However, no effect on the association of bFGF with CSR was observed when the HSPG were removed by heparinase treatment (Figures 1 and 4). This result suggests that binding of bFGF to the HSPG is not a required step for bFGF interaction with its CSR. While a small amount of HSPG may have been resistant to the heparinase digestion and participated in bFGF CSR binding, our conclusions would not change dramatically. The heparinase treatment significantly removed HSPG, decreased CSR equilibrium binding, increased the CSR K_d, and only altered the CSR *off* rate and not the *on* rate. Thus, the possibility that HSPG binding of bFGF conformationaly changes the growth factor such that it binds its receptor is unlikely but cannot be ruled out. It is interesting to note that a mechanism for cooperative "high-affinity" binding consistent with the kinetic constants does not require an HSPG-mediated alteration of either bFGF or CSR.

A model involving a "high-affinity" ternary complex is consistent with the "high-affinity" binding component being less susceptible to the high-salt wash than the bFGF–HSPG complex. However, in the presence of the high-salt solution, the ternary complex might be expected to dissociate with kinetics similar to those of the CSR alone (Figure 5) since the bFGF–HSPG interaction would be destabilized. Thus, the use of prolonged incubations (~10 min) with high-salt solutions to evaluate bFGF binding might result in the dissociation of a considerable amount (~20%) of the CSR-bound bFGF, and cause underestimation of the presence of these sites.

The approach described in this paper distinguishes between HSPG and CSR, yet no distinction has been made regarding the location of the HSPG. While it may be easier to envision the cell surface localized HSPG interacting with the CSR, the possibility that the HSPG in the ECM participates in bFGF binding to the CSR cannot be excluded since the ECM is intimately associated with the cell surface. At our level of understanding, a "high-affinity" ternary complex would only require that both the CSR and HSPG be effectively immobilized and close to one another. The isolated HSPG produced by solubilizing cells with Triton/NH_4OH might contain all, a fraction, or none of the plasma membrane intercalated HSPG. Subendothelial ECM isolated in this way has been demonstrated to contain bFGF binding HSPG with no other bFGF binding components (Bashkin et al., 1989). Finally, the binding of bFGF to HSPG on intact cells and isolated HSPG was almost identical (K_d = 0.76 vs 1.03 nM), suggesting that the HSPG in the Triton/NH_4OH extract was similar to that on intact cells.

Yayon et al. (1991) and Rapraeger et al. (1991) have demonstrated that the binding of bFGF to its receptor in heparan sulfate deficient cells can be recovered by the addition of soluble heparin or heparan sulfate. The results presented here suggest that soluble heparin would not function in a cooperative manner with the CSR to produce a high-affinity complex but instead would compete for cell-associated binding sites. However, heparin has been shown to bind specifically to the surface of many cell types (Glimelius et al., 1978; Kjellén et al., 1980; Castellot et al., 1985; Biswas, 1988); thus, the addition of heparin in soluble form to cells might result in the establishment of cell-associated heparin which might replace HSPG.

The extracellular matrix has been proposed to act as a storage site for bFGF in vivo (Vlodavsky et al., 1987; Baird & Ling, 1987; Folkman et al., 1988; Bashkin et al., 1989; Flaumenhaft et al., 1989). Heparin and endothelial cell-derived heparan sulfate bind and protect bFGF from inactivation (Gospodarowicz & Cheng, 1986; Saksela et al., 1988). The rapid *off* rate ($t_{1/2}$ = 5–10 min) of bFGF from HSPG on intact cells or isolated extracellular matrix at first seems to conflict with a role for long-term storage and stabilization of bFGF. However, the *off* rate measurements (Figures 2 and 5) represent the dissociation of individual bFGF–HSPG complexes since the experimental conditions were set up to prevent rebinding of released bFGF to HSPG. In the absence of a restraint on rebinding, the HSPG might act as high-frequency bFGF binding sites which bind, release, and rebind bFGF, providing a dynamic storage system for bFGF. Indeed, when the dissociation kinetics of bFGF from HSPG or CSR were measured in the absence of a restraint on rebinding, the observed rate of release was significantly slower ($t_{1/2} \simeq$ 250 and 800 min for HSPG and CSR, respectively). This type of storage mechanism might have several advantages over a static system of storage. For instance, the exposure of bFGF-containing ECM to bFGF-deficient environments could create a diffusion gradient which might result in release of bFGF from the ECM to the areas of low bFGF concentration without physical degradation of the ECM. In addition, this may serve to increase the capacity of bFGF storage by maintaining more bFGF molecules in the vicinity of the ECM.

The concept that growth factors interact with cells through single bimolecular binding with cell surface receptors seems to be an overly simplistic one. Recently it has become clear that the binding of bFGF to cell surface receptors and to heparan sulfate sites is coupled (Yayon et al., 1991; Rapraeger et al., 1991). Furthermore, the presence of heparan sulfate is required for bFGF to support the growth of Swiss 3T3 fibroblasts and suppress the terminal differentiation of MM14 skeletal muscle cells (Rapraeger et al., 1991). bFGF is one

of a growing number of growth factors that bind to heparin, and the possibility that the duel binding site system, consisting of receptor and glycosaminoglycan, is a general mechanism for growth factor–cell interaction has been suggested (Klagsbrun & Baird, 1991; Rouslahti & Yamaguchi, 1991), but not completely elucidated.

In this report, we measured the association and dissociation kinetics of bFGF with CSR and HSPG sites on Balb/c3T3. These measurements were made with nontreated cells, with cells digested with heparinase to remove the heparin-like bFGF binding component, and with detergent-extracted HSPG without CSR to determine the relative dependence of these binding sites on one another. We found that the difference in K_d's for the two affinity classes of bFGF binding resulted primarily from a difference in k_{off}'s and not k_{on}'s and that bFGF binding seemed to be controlled by HSPG as a result of stabilization of the bFGF–CSR complex, producing a "high-affinity" interaction. These results suggest a mechanism of protein–GAG cooperativity which involves the formation of a ternary complex of bFGF–CSR–HSPG that is more stable than either bFGF binding to CSR or HSPG alone. The dependence of the CSR K_d on the amount of HSPG might also help explain the wide range of K_d's reported for the CSR in different cells under different conditions [see Burgess and Maciag (1989) for a review]. It will be important to elucidate the chemical nature of this GAG/protein interaction and determine under what conditions this cooperative interaction occurs. This is especially important when one considers the large structural variability of proteoglycan molecules in different tissues, species, and states of cellular differentiation (Dietrich et al., 1983; Nader et al., 1987; Kjellén & Lindahl, 1991). Thus, the importance and complexity of GAG-controlled growth factor action are only beginning to become appreciated.

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